Remarks

Claims 24-26 are canceled herein. Claims 1, 3-10, 12, 13 and 22-23 and 27-29 are pending in this application following entry of this amendment. Claim 1 is amended herein. Support for the amendment of claim 1 can be found throughout the specification, such as, but not limited to, page 41 and pages 105-114. New claims 27-29 are added herein. Support for new claims 27-29 can be found throughout the specification, such as but not limited to pages 105-114 and FIGS. 14-18.

No new matter is added herein. Reconsideration of the application is respectfully requested in view of the foregoing amendments and following remarks. Applicants believe that the application will be in condition for allowance following entry of this amendment.

Rejections under 35 U.S.C. § 103(a)

Claims 24-26 are rejected under 35 U.S.C. § 103(a) as allegedly being obvious over Casanova et al. in view Lee et al. Claims 24-26 are also rejected under 35 U.S.C. § 103(a) as allegedly being obvious over Casanova et al. in view of Stewart et al. Applicants respectfully disagree with this rejection. However, solely to advance prosecution, in order to obtain a more rapid allowance of the other pending claims, claims 24-26 are canceled herein. Applicants respectfully disagree with the rejection, and expressly reserve the right to pursue claims 24-26 in a continuation application.

Claims 1, 3-10, 12-13 and 22-23 are rejected under 35 U.S.C. § 103(a) as allegedly being obvious over Rajewsky et al. in view of Lee et al.

Rajewsky et al. disclose that the genome in ES cells can be modified by conditional gene targeting using the Cre-loxP system. Rajewsky et al. describe a gene targeting vector including three loxP sites can be generated. This targeting vector includes three loxP sites, two of which flank a nucleic acid encoding a selectable marker. Rajewsky et al. state "the strategy of conditional targeting of the endogenous genes that we have developed consists of flanking a target gene segment with loxP sites in ES cells by *classical gene targeting..*" [emphasis added, see page 601, second column]

Rawjewsky et al. teach that the targeting vector is introduced into embryonic stem cells. The endogenous homologous recombination of ES is used. The result is that the selectable marker flanked by two loxP sites and the additional loxP site is introduced into the genome of the host ES cell. The expression of Cre in the ES cells results in recombination at the loxP sites. In some (but not all) of the

ES cells, the selectable marker is deleted, and two loxP sites remain. These ES cells are then used to generate mice. The mice including the two loxP sites are mated with another mouse that expresses Cre in specific cells of interest.

In the system described by Rajewsky et al. appropriate positioning of the LoxP sites can result in mutagenesis and knock-outs. However, this only occurs when mice carrying two loxP sites inserted into the gene of interest in the mouse chromosome are mated to a second strain of mice that express the Cre recombinase.

Rajewsky et al. do not disclose any specific methods, such as those claimed, for generating conditional knockout vectors that utilize homologous recombination. There is no information in Rajewsky et al. with regard to methods for producing the vectors that are shown in Figure 1. There is most certainly no mention or suggestion in Rajewsky et al. of a bacterial artificial chromosome (BAC) to produce the vector, let alone introducing a first selectable marker flanked by a pair of first recombining sites into a BAC, as required by claim 1. In addition, there is no mention or suggestion to use two pairs of recombining sites (as even the final vector only includes three identical recombining sites). There further is no mention in Rajewsky et al. that a first selectable marker is excised during the generation of the targeting vector. Indeed, in the methods disclosed in Rajewsky et al. the only selectable marker is excised using Cre after the introduction of the final targeting vector into ES cells.

Rajewsky et al. suggest that classical gene targeting is effective at producing the vectors. Seibler et al. (Nucl. Acids Res. 31: e12, 2003; of which Rajewsky is an author) describes the method used to produce the constructs described by Rawjesky et al. Classical methods, such as PCR amplification, restriction digestion, ligation and mutagenesis are used to produce the vectors used by Rajewsky et al. Seibler et al. state that the derivation of conditional mouse mutants "has been a time consuming undertaking" involving three breeding steps of three months duration each (see page 7 and FIG. 5A), that is required to obtain mice combining two transgenes. Thus, it is clear that the methods utilize by Rajewsky et al. involve classical cloning methods and animal husbandry.

Interestingly, Seibler et al. propose introducing loxP sites in ES cells carrying a Cre gene, so that mouse mutants can be derived directly from ES cells. LoxP sites are introduced individually into both alleles of the target gene. In addition, a vector is used that has a FLP-flanked **single** selection marker. Clearly, Seibler et al. teach away from using both LoxP sites and FLP sites, or two selection markers, in the targeting vector itself, as they show it is desirable to introduce recombining sites

directly into the gene in the chromosome. This new strategy allows the production of a mouse in six months.

Rajewsky et al. simply cannot be construed to suggest the claimed methods for producing gene targeting vectors that utilize homologous recombination in bacterial cells, let alone the introduction of a second nucleic acid encoding a second selectable marker flanked by a pair of second recombining sites into a gene in a bacterial artificial chromosome, let alone suggest excising the nucleic acid encoding the second selectable marker with a second recombinase specific for the second recombining sites, such that two first recombining sites remain in the gene following excision of the nucleic acid encoding the selectable marker.

The only homologous recombination used in the methods disclosed in Rajewsky et al. (or Seibler et al.) is the *endogenous mechanisms of recombination that take place in ES cells*, wherein the a vector is incorporated into the genome. Thus, this reference cannot in any way be construed to suggest, nor render obvious, the use of homologous recombination is in bacterial cells for the production of vectors, as specified in claim 1.

The Office action concedes (see page 12 of the Office action) that Rajewsky et al. do not teach two steps to introduce loxP sites, nor does it teach the use of homologous recombination to insert any nucleic acids into bacterial artificial chromosomes, let alone a first selectable marker flanked by a first pair of recombining sites and a second selectable marker flanked by a second pair of recombining sites. The Office action further concedes that Rajewsky et al. do not teach the use of a de-repressible promoter operably linked to a nucleic acid encoding Beta, Exo and Gam. Thus, it is unclear what would lead one of skill in the art to combine Rajewsky with any reference disclosing recombineering in bacterial cells.

Lee et al. teach a de-repressible promoter (pL) operably linked to a nucleic acid encoding Beta, Exo and Gam under the control of a temperature sensitive repressor. Lee et al. teach that this method can be used to introduce the arabinose promoter operably linked to Cre, and describe the introduction of a FRT-Kan-FRT cassette into a gene in a bacterial artificial chromosome. However, Lee et al. do not suggest, or render obvious inserting a second nucleic acid encoding a selectable marker flanked by a pair of second recombining sites into a second site in the gene. In addition, Lee et al. do not suggest, nor render obvious, excising the nucleic acid encoding the selectable marker with a second

recombinase specific for the second recombining sites, wherein two first recombining sites remain in the gene following excision of the nucleic acid encoding the selectable marker, and wherein recombination of the two first recombining sites produces a nucleic acid sequence that cannot be transcribed to produce a functional protein.

Submitted herewith is the Declaration of E-Chiang Lee (the first author of Lee et al.) and Pentao Liu under 37 C.F.R. § 1.132 (hereinafter the Declaration). The Declaration describes the work performed by Drs. Lee and Liu. The Declaration delineates the dramatic differences between Lee et al. and the presently claimed methods, namely the methods described in Lee et al. do not use a second set of recombining sites flanking a second selectable marker, and they do not provide any information on how to produce a nucleic acid sequence that **cannot be transcribed into a functional protein**. Indeed, the intent of Lee et al. is to induce a nucleic acid encoding functional Cre protein into an untranslated region of a gene (Eno2), such that Cre is expressed in a tissue specific manner (along with the Eno2 protein). Thus, Lee et al. describes method for introducing a functional gene in a regulatory element so that the encoded protein is properly expressed. The methods described in Lee et al. are completely opposite from the presently claimed methods of generating vectors for the knockout of a gene, wherein recombination of the recombining sites produces a nucleic acid sequence that cannot be transcribed to produce a functional protein, as required in claim 1.

Furthermore, there are missing elements in the combination of Rajewsky et al. and Lee et al. These references, even in combination, simply do not suggest: (1) the use of two selectable markers, each flanked by a pair of recombining sites; (2) the use of homologous recombination to make vectors for conditional knock-outs; or (3) specific steps in any method that would lead to a vector for a conditional knockout. A *prima facie* case of obviousness cannot be established when the proposed combination does not include all of the elements of the claim.

The Declaration refers to the data presented in the previous Declaration of Dr. Liu under 37 C.F.R. § 1.132, which is already of record. As a secondary consideration, the Applicants note that *a large number of experiments were required to devise the presently claimed methods*. Dr. Lee was not able to simply use the work he presented in Lee et al. to quickly devise methods for producing vectors for conditional knockouts. The large number of the required experiments is evidenced in data and methods presented the Declaration of Dr. Liu under 37 C.F.R. § 1.132. Applicants request that the Office consider this evidence as a secondary consideration, see MPEP § 2145.

Claims 1, 3-10, 12-13 and 22-23 are rejected under 35 U.S.C. § 103(a) as allegedly being obvious over Rajewsky et al. in view of Muyers et al. and Stewart et al.

Rajewsky et al. is described above.

Muyers et al. describe the use of antibiotic –resistance genes, and homologous recombination, to produce BACs, P1-vectors, or PACS, or mouse knockout constructs, see pages 325-326. However, the methods of Muyers et al. are very different from the presently claimed methods. Specifically, Muyers et al. states that "[i]n the first round, homologous recombination is used to generate an initial product by the integration of the of the selectable gene, together with additional functional elements, at the intended site. In the second round, the extra functional elements are used to remove the selectable gene, thereby generating the final product." Thus, the "two rounds" of processing described in the Office action on page 14 is one step ("round") of recombination, followed by one step ("round") of the use of a recombinase. At page 326, Muyers et al. further describes one step of using homologous recombination, followed by a second step of the use of a recombinase or restriction enzyme. Thus, the "two rounds' referred to in the Office action are very different than the steps in the presently claimed methods. Interestingly, when describing the use of recombining sites (termed "site specific recombination target sites" or "SSRTs" in Muyers et al.), Muyers et al. state "the remaining 34 (or 36) bp scar (the SSRT) can be a problem if left in protein coding, or regulatory, regions." *Thus, Muyers et al. teach away from the use of recombining sites.*

Stewart et al. and Muyers et al. teach alternative methods for homologous recombination in bacterial cells, describing cloning and subcloning and documenting that homologous recombination can be used to introduce a single selectable marker. *There is no description in either Stewart et al. or Muyers et al. of any specific steps in a method for generating conditional targeting vectors.* Neither of these references describes: (1) the use of two selectable markers, each flanked by a pair of recombining sites; (2) the use of homologous recombination to make vectors for conditional knock-outs; or (3) specific steps in any method that would lead to a vector for a conditional knockout. Thus, neither Stewart et al. or Muyers et al. make up for the deficiencies of Rajewsky et al. A *prima facie* case of obviousness cannot be established when the proposed combination does not include all of the elements of the claim.

The lack of specific steps described in the cited prior art makes it very difficult for the

Applicants to provide a comparison of methods to document the unexpected superior properties of the claimed methods. The claimed methods utilize recombineering with Beta and Exo (as recited in claim 1) rather than restriction enzymes and DNA ligases for vector construction (see the specification, Fig. 18). By using high copy plasmid DNA for vector construction, the problem caused by Lox sites present in the BAC vector backbone is eliminated (discussed in Rajewsky et al. and Muyers et al.). The Declaration, and the data presented in the specification, documents that as many as 10,000 colonies can be obtained from a single subcloning experiment with only 50-100 ng of retrieving plasmid DNA. In addition, more than 95% of the colonies are correctly constructed. Moreover, using long homology arms, targeting frequencies as high as $1X10^{-2}$ can be obtained with as little as 100 ng of targeting DNA (such as when inserting a floxed *Neo* cassette to a BAC).

The Declaration further documents that classical molecular techniques generally take several months to prepare a vector. However, the presently claimed methods allow one person to make a conditional knock-out vector in 2-3 weeks (see, for example, pages 108-111 of the specification). The efficiency of the claimed methods simply cannot not be predicted based on Lee et al., Muyers et al., Rajewsky et al. or Stewart et al., alone or in any combination. The documentation of the extreme efficiency of the claimed methods overcomes any prima facie case of obviousness.

Reconsideration and withdrawal of the rejections are respectfully requested.

Conclusion

Applicants believe that the present claims are in condition for allowance, which action is requested. *If any issues remain prior to allowance, the Examiner is formally requested to contact the undersigned prior to issuance of the next Office action, in order to arrange a telephonic interview.* It is believed that a brief discussion of the merits of the present application may expedite prosecution. This request is being submitted under MPEP §713.01, which indicates that an interview may be arranged in advance by a written request.

Respectfully submitted,

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